PRODUCTION OF ANTIBODY AGAINST GLOMALIN FROM THE 32B11 CELL LINE

(Goding, 1986; Hurrell, 1982; Zola, 1987)

Introduction

The hybridoma for production of the antibody against glomalin (32B11) can be purchased from ATCC (American Type Culture Collection). The catalog number is CRL-2559 and the cost is $290.00. The cell line should be stored in liquid N_2. After culturing, the growth media containing antibodies may be used in ELISA, immunofluorescence, and dot blot assays to quantify and/or examine glomalin.

Culture Media (RPMI media supplemented with 10% Fetal Calf Serum)

Materials

Note: All reagents including the water must be tissue culture grade. Makes 500 ml.

8.2 g RPMI media (HEPES)*
0.03 g penicillin**
0.066 g streptomycin**
0.055 g sodium pyruvate
1.0 g sodium bicarbonate
400 ml tissue culture grade water (T 15-20 ^{o}C)
50 ml FCS (Fetal Calf Serum) for 10% FCS in final solution***
Sterivex (GV 0.22um filter unit) (Millipore Catalog No. SVGVB1010)
150 cm^2 (500 ml) tissue culture flasks (preferably with 0.22 um filters in top)

*Glutamine in RPMI media has a short half-life. If you are storing media longer than 15 days, add 0.30 g/L of L-glutamine by suspending L-glutamine in as small amount of DD H_2O as possible then filter into flask before use. Note: It is best to use media within a few days.

**Antibiotics prevent bacterial growth in the media, but sterile techniques must be adhered to or the sample will become contaminated by fungi. This is a very nutrient-rich media and is easily contaminated.

***FCS must be kept in the dark (light can result in the formation of toxins). FCS will have some insoluble material in it, but if it looks cloudy, you will have to filter before using (changing filters frequently).

Methods

1) Rinse graduated cylinder or beaker and stir bars thoroughly with Milli-Q water before making media.
2) Combine #1-6 and add cold* tissue culture grade water until volume reaches 450 ml. Mix completely. See Fig. 1.
3) Filter sterilize through a 0.22 μm filter unit using a peristaltic pump into sterile tissue culture flasks. See Fig. 2. Sterile techniques must be adhered to including working under a laminar flow hood with gloves and sterile sleeves for your arms.
4) Sterilize outside of FCS bottle with 70% ethanol from a spray bottle prior to opening and adding to the media.
5) Add appropriate amount of FCS. Refrigerate until needed.

*Water must be 15-20^{o}C to dissolve some of the amino acids. See Fig. 1B.

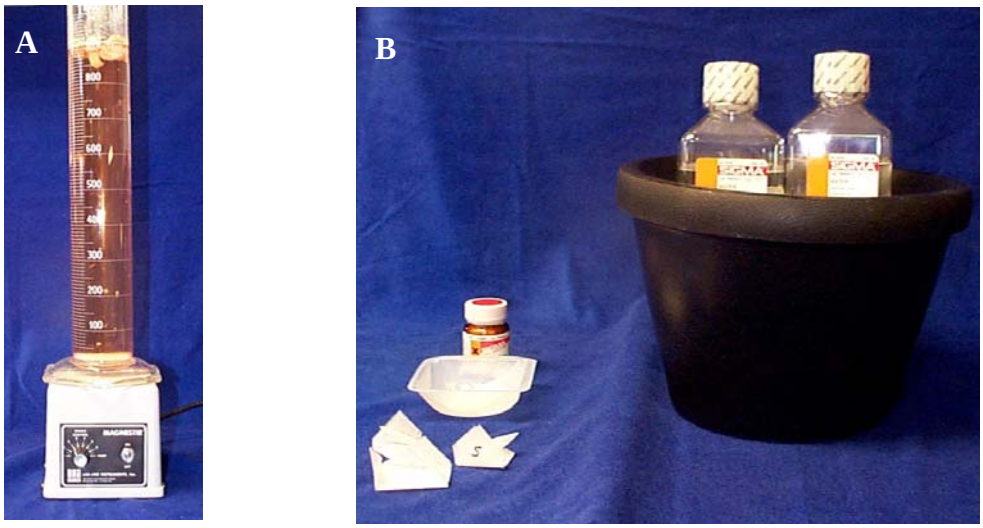

Figure 1. Dry ingredients (all except for the Fetal Calf Serum) in the RPMI media are mixed together (A) and brought to volume with 15-20^{o}C tissue grade water (B), which also helps to dissolve some of the amino acids.

Figure 2. The RPMI media is filtered through a 0.22 um filter into a tissue culture flask before adding the Fetal Calf Serum.

Antibody Production

Materials

Very clean, sterile glass bottles or 25 cm^2 (approx. 50 ml) tissue culture flasks
Very clean, sterile glass bottles or 150 cm^2 (approx. 500 ml) tissue culture flasks
Water-jacketed CO_2 incubator$_r$ with temperature and gas regulation
50 ml centrifuge tubes

Methods

1) Clean incubator using 70% ethanol from a spray bottle.
2) Using a sterile, disposable pipette, remove 10 ml of RPMI media and transfer to a 25 cm^2 flask.
3) Remove cryo-vial containing 32B11 cell line from liquid N_2 and place immediately into a plastic dish (with a cover) containing water at 37^{o}C. Cover immediately. See Fig. 3. The vial may bounce around and could fly up and injure someone if not covered. Wait until contents defrost, spray the outside of the vial with 70% alcohol, remove cap and pour into the 25 cm^2 flask with RPMI media.
4) Place in incubator with slightly slanted shelves so flasks will not leak. See Fig. 4. Place a sterile tray in the bottom about 1/3 full of sterile (autoclaved) Milli-Q water (change water every 3 to 4 days). Flasks must have their caps loose or have caps with 0.22 μm filters to allow gas exchange while maintaining sterile conditions. Incubator must be set at 37^{o}C with 5% CO_2 (regulator at 10 psi).
5) **Scale-up phase**. We produce 1 to 2 liters of antibody solution each time we culture cells. A scale up from the original culture of the hybridoma is necessary, and this takes a few weeks. Observe cells placed in the 25 cm^2 flask by using an inverted

microscope after 2 to 3 days of growth. Healthy cells will be bright, smooth and shiny spheres. Dead cells appear dull and have a rough surface. When cells cover about 2/3 of the microscopic field of view, transfer 1 ml into a new 25 cm^2 flask with 10 ml of media (or all 10 ml into a larger 150 cm^2 flask with 30-40 ml of media).

6) Add new media to the original flask until volume is 10-12 ml. Check growth every day to be sure that cells do not become overcrowded during this scale-up phase of culturing. When cells cover 2/3 of the microscopic field, split the culture (half to a new flask with an equal amount of growth media, and replenish the original flask with growth media). Keep splitting and adding growth media until you have used all the growth media. You will have five or six 250 cm^2 flasks with 150 to 200 ml of growth media. Do not overfill flasks and do not allow media to touch the lid.
7) If you wish to store cells for future use, the cells that grow from the first culture of a cell line clone are the best ones to use. It is important that cells are healthy and have not been repeatedly subcultured in order to maintain viability during the freezing process and to prevent overgrowth of a mutation that could be a non-producer of antibodies. Consult any of the numerous books on hybridoma production for freezing techniques.
8) **Antibody production phase**. Let cells overgrow until they die. Media will change color slightly, and after shaking the flask gently to suspend the cells, the media will look cloudy. Check cultures daily for fungal growth. You may be able to save the remaining flasks if you catch fungal growth in only one flask at an early stage.
9) After cells have died, transfer growth media to sterile (new) centrifuge tubes and centrifuge at 3000 × g for 5 min.
10) Pour supernatant (antibody solution) into sterile (new) 50 ml containers. Centrifuge tubes or flasks work the best, because you can dispense easily from these tubes. Store in refrigerator (this antibody precipitates at –20 ^{o}C, and therefore, cannot be stored in the frozen state.)
11) Dispose of pelleted cells.
12) Use a serial dilution to determine the antibody dilution factor appropriate for your antibody solution. We do not have a universal standard for glomalin at this time. We use a highly immunoreactive soil to make our standard. We use a 1:6 antibody solution:PBS dilution factor and get an OD_{405} for phosphatase ELISA ca. 3.0 reacted against 0.04 ug of glomalin. See ELISA instructions at this Internet site for further details about our ELISA method.
13) To prevent contamination, antibody from the 50 ml container in step 9 may be dispensed into 1.5 ml eppendorf tubes (enough for one ELISA plate). This eliminates the need to open and pipette from a large amount of antibody (i.e. 50 ml) several times.

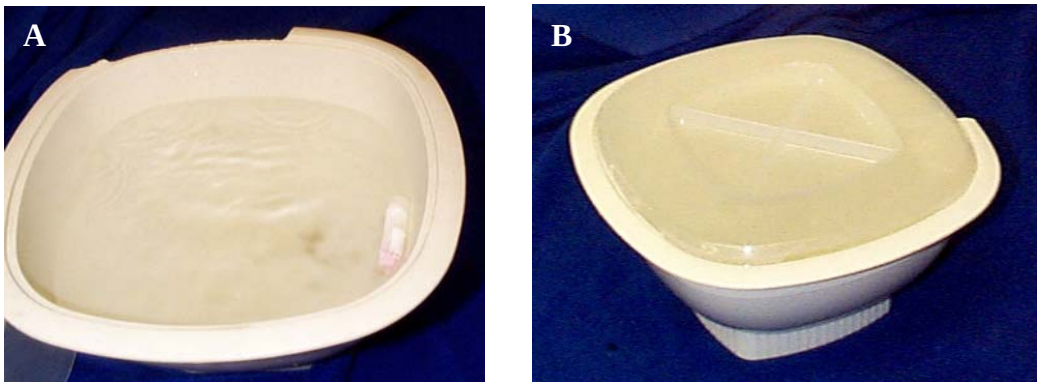

Figure 3. The cryo-vial is removed from liquid N_2 and immediately placed in water at 37^{o}C (A). The vial will bounce around as it rises to room temperature. Cover to prevent injury (B).

Figure 4. Tissue culture flasks are placed at an angle in an incubator at 37^{o}C with 5% CO_2 and sterile (autoclaved) Milli-Q water in a tray at the bottom.